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Characterization of rotavirus specific B cells and their relation with serological memory

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ABSTRACT

We quantified circulating total, rotavirus (RV) and Tetanus toxin (TT) memory B cells (mBc) in healthy adults using a limiting dilution assay (LDA) and a flow cytometry assay (FCA) that permit evaluation of both CD27+ and CD27− mBc.

RV mBc were enriched in the CD27−, IgG+ and in the CD27+, IgM+ subsets. The numbers of RV mBc were higher by FCA than by LDA and results of the two assays did not correlate. TT IgGmBc and RV IgA mBc determined by FCA and by LDA correlated with TT plasma IgG and RV plasma IgA, respectively. The mean ratio of specific mBc/μg/ml of the corresponding plasma immunoglobulin was lower for TT IgG than for RV IgA mBc.

Our studies contribute to understand the relationship between circulating mBc and serological memory, and enhance our capacity to develop better correlates of protection against RV disease.

Introduction

Rotavirus (RV) is the principal etiological agent of severe gastroenteritis (GE) in young children worldwide (Parashar et al., 2006). Despite their preferential replication in the intestine, RV induces both an intestinal and systemic immune response, the latter most probably related to the antigenemia and viremia present in acutely infected children and animals (Blutt and Conner, 2007; Blutt et al., 2007). The RV-serum IgA titer, measured shortly after natural infection or oral vaccination, is a relatively good correlate of protection (Angel et al., 2007; Franco et al., 2006). However, our understanding of immunity induced by different RV vaccines is poor and, in general, good correlates of protection following vaccination have not been found (Angel et al., 2007; Franco et al., 2006; Rojas et al., 2007). As part of an effort to identify better correlates of protection for RV vaccines, we and others have developed a flow cytometry assay (FCA) to quantify and characterize RV-specific B cells in mice (Feng et al., 2006; Youngman et al., 2002), and naturally infected and vaccinated children (Gonzalez et al., 2003; Jaimes et al., 2004; Parez et al., 2004; Weitkamp et al., 2005). Using this assay, RV binding B cells seem to be a unique subset: as expected, shortly after acute RV infection a fraction of RV-memory B cells (mBc) express intestinal homing receptors (Jaimes et al., 2004). Moreover, it was recently shown that VP6 RV protein interacts specifically with a large fraction of naive B cells (CD27−) from both adults and neonates (Parez et al., 2004). The naive and mBc that bind RV VP6 predominantly express the VH1–46 gene segment and are enriched in the CD27+, IgD+, IgM+, subset (Tian et al., 2008).

A thorough understanding of the frequencies of antigen specific mBc subsets and their relationship with the antibodies in serum (serological memory) is likely to be critical to identify useful correlates of protection for vaccines (Hofer et al., 2006; Lanzavecchia et al., 2006; Nanan et al., 2001; Sasaki et al., 2007). Using tetanus toxin (TT) as a model antigen, the number of specific mBc have correlated with some, but not other, specific serum TT antibody concentrations, depending on the types of assays used. Using a functional assay, in which blood lymphocytes were stimulated polyclonally and TT-specific mBc were quantified by limiting dilution assay (LDA), this correlation was found (Bernasconi et al., 2002; Lanzavecchia et al., 2006). On the other hand, the quantity of TT mBc, measured by a FCA similar to the one we have previously used for RV mBc, did not correlate with the levels of serum TT-specific IgG (Leyendeckers et al., 1999).

The purpose of the present study was to quantify and compare the frequencies of circulating RV IgM, IgG and IgA mBc using LDA and FCA in healthy adults, and to establish if there was a correlation between the numbers of RV-specific IgG and IgA mBc, detected by each method, and the corresponding plasma RV antibody levels. For comparison, total (non antigen specific) and TT-specific mBc levels were evaluated.

Results

Frequencies of total and antigen specific mBc measured by LDA

To determine the most sensitive method to evaluate RV-specific mBc by LDA, we first compared the frequency of these mBc detected

Abbreviations: RV, rotavirus; Ig, immunoglobulin; mBc, memory B cell; TT, tetanus toxin; LDA, limiting dilution analysis; FCA, flow cytometry assay.

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after stimulating PBMC or purified B cells using different published protocols (Amanna and Slifka, 2006; Bernasconi et al., 2002; Crotty et al., 2004), or minor variants of these protocols described in Materials and methods. As shown in Fig. 1a, the number of RV-specific IgM/IgG/IgA mBc detected in PBMC stimulated with CpG and IL-2 were higher or comparable to those obtained with the other methods. We thus selected the IL-2 and CpG stimulation of PBMC to determine the frequency of circulating total, TT and RV-specific mBc by LDA. Fig. 1b shows an example of these experiments. Less than 199 total antibody producing B cells per 10^6 B cells were present in unstimulated PBMC. Results for individual volunteers and median frequency of total and antigen specific IgM, IgG and IgA mBc are reported in Fig. 2. The frequency of total IgG and RV IgG mBc detected by LDA was higher than that of total IgM and RV IgM mBc (Wilcoxon, $p<0.017$). In contrast, the TT IgM mBc detected by LDA were more frequent than the TT IgG mBc (Wilcoxon, $p=0.012$). Thus, using LDA, most total mBc seem to bear the IgG isotype, but for individual antigen specific mBc either IgG (RV) or IgM (TT) can be the predominant isotype.

Frequencies of total and antigen specific mBc measured by FCA

To evaluate the frequency of total and antigen specific mBc detected by FCA, we purified CD19+ B cells with microbeads and stained them with antibodies against CD20 (a marker of B cells, except ASC), CD27 (a marker of most (Klein et al., 1998) but not all mBc (Fecteau et al., 2006; Wirths and Lanzavecchia, 2005)) and IgM, IgG or IgA to identify different isotypes of mBc. To recognize RV mBc we used fluorescent RV VLPs (Jaimes et al., 2004; Rojas et al., 2007; Youngman et al., 2002) and to detect TT mBc we used biotinylated TT followed by labeled streptavidin (Amanna and Slifka, 2006). For analysis, we considered both the CD27+, IgM, IgG or IgA classically described mBc (Klein et al., 1998) and the more recently described CD27−, IgG or IgA mBc (Fecteau et al., 2006; Wirths and Lanzavecchia, 2005). Fig. 3 shows a representative FCA experiment to evaluate CD20+, CD27+ (Fig. 3a) and CD20+, CD27− (Fig 3b), naive and mBc.

Results of IgM, IgG and IgA mBc from all volunteers, detected by FCA (the sum of CD27+ and CD27− subpopulations), are summarized in Fig. 2, where they are compared to the results obtained with the LDA. In contrast with the results by LDA, the median total, TT and RV IgG mBc measured by FCA tended (Wilcoxon, $p>0.1$) to be slightly lower than their respective IgM mBc. The frequencies of total IgG, IgM and IgA, TT-specific IgG and RV-specific IgM, IgG and IgA mBc were higher by FCA than by LDA (Wilcoxon $p<0.03$) (Fig. 2). This difference seems especially important for IgA mBc and future studies are necessary to establish if conditions other than those used in Fig. 1a will improve the efficiency of the LDA for these

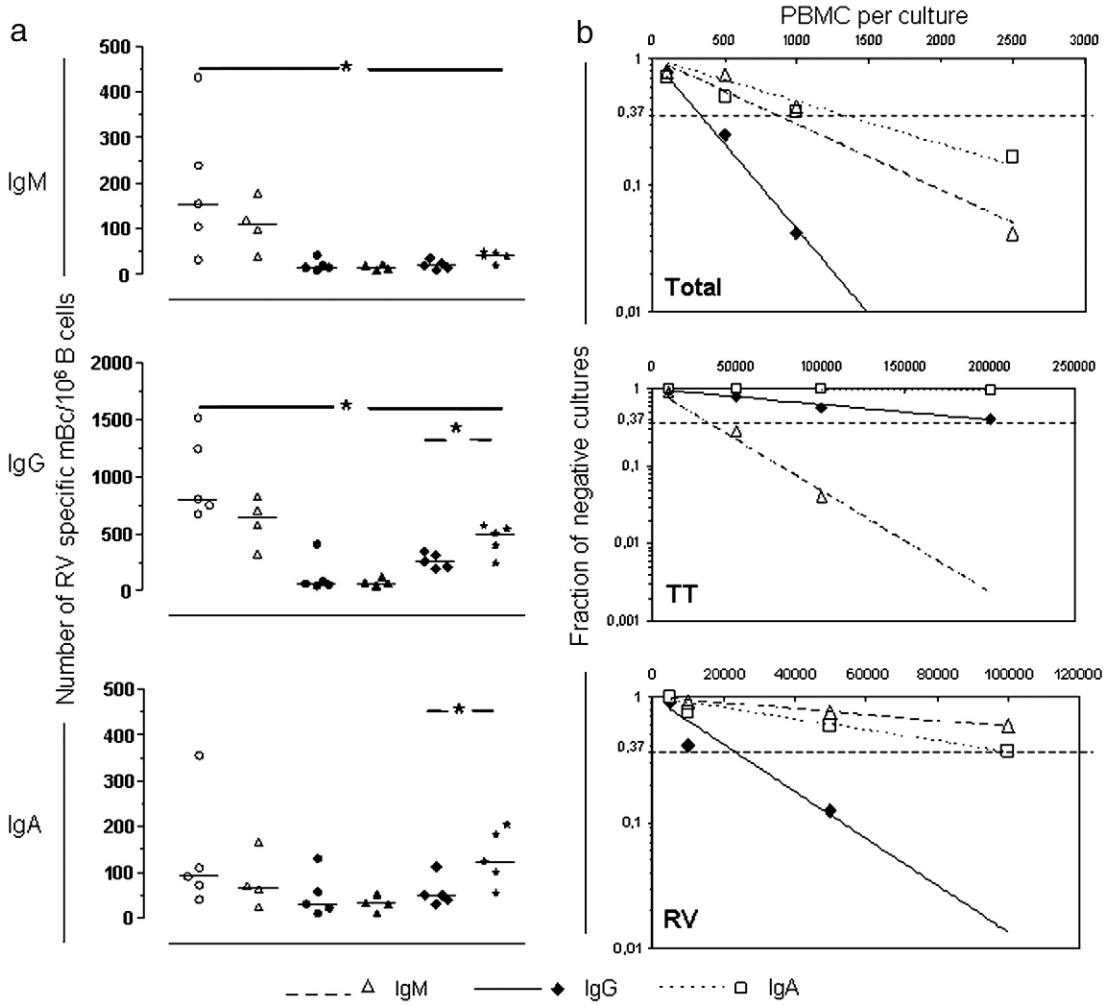

Fig. 1. (a) Higher or comparable numbers of RV IgM/IgG/IgA mBc were detected in CpG+IL-2 stimulated PBMC than with other stimulation methods. PBMCs (empty symbols) or purified B cells (filled symbols) were polyclonally stimulated with CpG+IL-2 (circles), CpG+SAC+PWM (triangles), CpG+IL-2+ autologous CD4+ T cells (diamonds) or CpG+IL-2/6/10+NIH/3T3 murine fibroblasts as feeder cells (stars). Specific mBc precursors were detected by ELISA after 10 days of culture. Bars represent the median. *Statistical differences by Mann Whitney test $p<0.05$. (b) Representative LDA experiments to determine total, TT and RV-specific mBc in PBMC polyclonally stimulated with CpG+IL-2, as described in Materials and methods. mBc frequencies were calculated from the 37% intercept. Correlation coefficients (R^2) were >0.75 and $p<0.05$, except for the TT IgA mBc that were below the sensitivity of the assay.

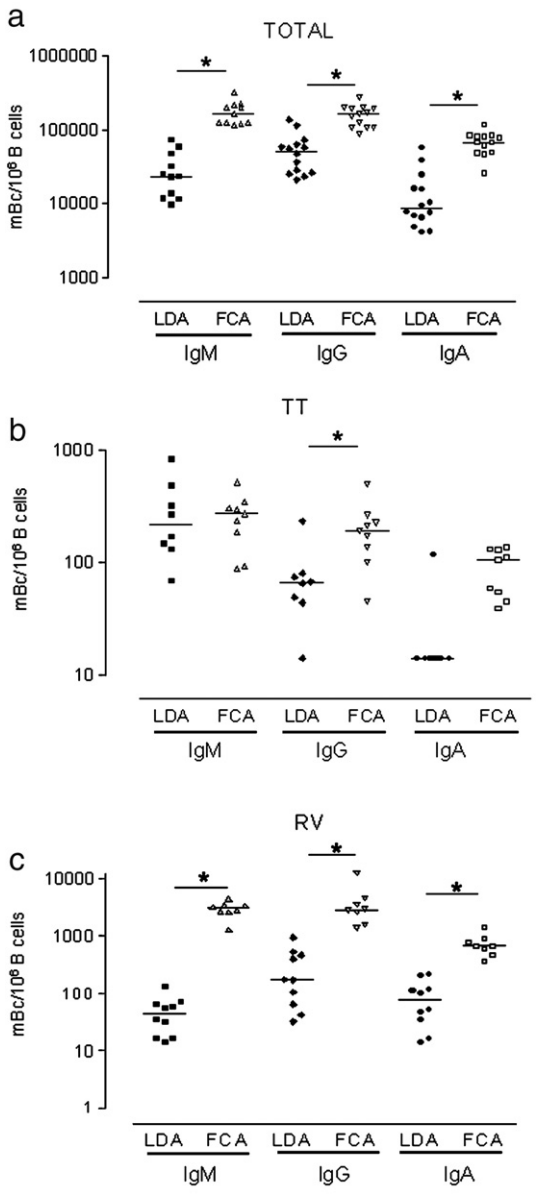

Fig. 2. Frequencies of mBc were determined by LDA and FCA (analyzing CD27+ and CD27− mBc subsets together), as described in Figs. 1 and 3 respectively. (a) Total IgM, IgG and IgA (n=11–14). (b) TT and (c) RV-specific IgM, IgG and IgA (n=8–10). Median values are represented by a line. *Significant difference (Wilcoxon test, $p<0.03$).

cells. In addition, levels of total and antigen specific mBc measured by LDA and FCA did not correlate (data not shown). Thus, results with both assays differ quantitatively and qualitatively, and this finding suggests that they measure overlapping but distinct populations of mBc.

We next set to determine the relative contribution of each major FCA mBc subset (CD27+/IgM+, CD27+/IgG/IgA+, CD27−/IgG/IgA+) to total and Ag-specific mBc. For this analysis we considered the sum of the three types of mBc as 100% of mBc (Figs. 4a and b). We found that the percentage of RV-specific CD27−, IgG/IgA+ mBc was significantly higher than the percentage of total CD27−, IgG/IgA+ mBc (Wilcoxon p=0.012) (Fig. 4a). This was not true for TT CD27−, IgG/IgA+ mBc. Analysis of CD27−, IgG+ and CD27−, IgA+ mBc subsets independently showed that the enrichment of RV-specific CD27− mBc depends on the IgG isotype (Fig 4b). Recently, it has been determined that, when only CD27+ mBc are considered, RV mBc are enriched in the IgD+, IgM+ subset (Tian et al., 2008). Performing a similar analysis in CD27+ cells we obtained comparable results and this was not true for the TT CD27+, IgM+ subset (Fig. 4c).

Thus, RV mBc are enriched in CD27−, IgG+ and in the CD27+, IgM+ mBc subsets. Of note, and as previously reported (Parez et al., 2004), naive B cells (estimated as CD27−, IgM+ and CD27− IgG/IgA− Fig. 3b) seem to be the predominant population of B cells that bind VLPs. RV antigen does not seem to be unique in this aspect (Hangartner et al., 2006; Parez et al., 2004; Ueki et al., 1990).

Correlation between mBc frequencies and plasma antibody levels

Trying to understand the relationship between mBc and serologic memory, we determined if circulating mBc levels correlated with the respective concentration of specific plasma Ig. The median (range) of total and antigen specific plasma Ig concentrations of the individuals studied is shown in Table 1. We did not measure antigen specific IgM because we could not purify antigen specific Ig of this isotype to use as a standard in the ELISA.

A correlation was not observed between total IgM, IgG and IgA mBc detected by either FCA (when CD27+ and CD27− were analyzed together, or when either subpopulation was analyzed separately) or LDA and the corresponding total plasma Ig level (data not shown). In contrast, there was a significant correlation between the frequency of TT IgG mBc detected by LDA and the corresponding plasma antibody levels (Fig. 5a). However, a correlation was not observed between TT IgA mBc measured by LDA and TT plasma IgA (data not shown), possibly because the levels of IgA mBc were below the level of detection of the LDA in several volunteers. In addition, a correlation was found between the frequency of RV IgA mBc measured by LDA and RV plasma IgA level (Fig. 5a). This correlation was not found between RV IgG mBc and RV plasma IgG (data not shown p=0.3 and R^2=0.57). For CD27+ mBc determined by FCA, we found a correlation between the frequencies of TT IgG mBc and the plasma IgG level as well as between RV IgA mBc and the respective plasma IgA RV antibody level (Fig. 5b). However, when both CD27+ and CD27− mBc were considered together in the analysis, only the correlations between the frequency of TT IgG mBc and the corresponding plasma antibody levels were conserved (data not shown). Antigen specific CD27− mBc (analyzed independently) did not correlate with plasma antibody levels (data not shown).

The mean ratio of specific LDA mBc/μg/ml of the corresponding plasma Ig was lower for TT IgG (73.3) than for RV IgA (693.6). Similarly, the mean ratio of specific CD27+ FCA mBc/μg/ml of the corresponding plasma Ig level was lower for TT IgG (116.46) than for RV IgA (11,153). Thus, with both methods the ratio of specific IgA mBc/plasma IgA concentration seems to be higher than the corresponding IgG ratios.

Discussion

We have shown that RV mBc are enriched in the CD27+, IgM+ and in the CD27−, IgG+ mBc subsets. In addition, we found that the frequencies of RV mBc measured by FCA are higher than those measured by LDA, and shown that RV IgA mBc, quantified by both assays, correlate with the concentrations of RV plasma IgA. To our knowledge our study is one of the first to study human circulating antigen specific IgA mBc (Ueki et al., 1990), and the first to show that these cells correlate with IgA serological memory.

We found relatively high frequencies of RV mBc by FCA, (Fig. 2c), and, as recently described (Tian et al., 2008), RV mBc were enriched in the CD27+, IgM+ mBc subset (Fig. 4). Previous investigators have reported similar frequencies of RV CD27+ mBc (~5025/10^6 B cells) in healthy adult volunteers (Parez et al., 2004). Unlike the RV IgD− mBc, that are absent from naive mice (Youngman et al., 2002) and children (Gonzalez et al., 2003), it is unknown if the RV-2/6-VLP binding IgM mBcs we have detected in healthy adults are induced by prior RV

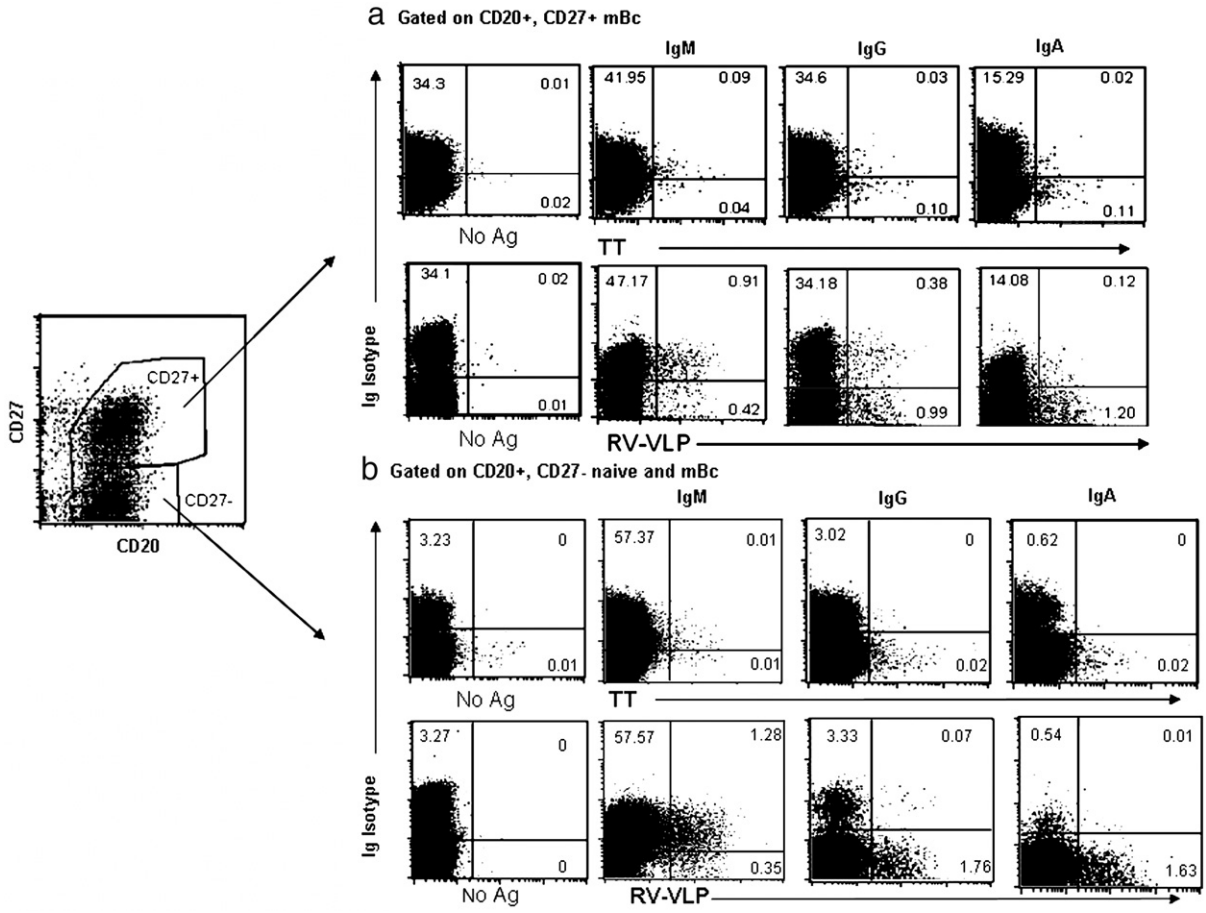

Fig. 3. Representative FCA dot plots of TT and RV-specific mBc from one volunteer. Purified B cells were gated on CD20+/CD27+ and on CD20+/CD27− subsets (left dot plot). On these gates we performed: (a) Analysis of the expression of IgM+, IgG+, IgA+ on CD20+/CD27+ mBc. (b) Analysis of the expression of IgM (naïve), IgG+ and IgA+(mBc) on CD20+/CD27− naive B cells and mBc. The first left dot plots in each row are controls stained with the anti-IgG antibody, and only streptavidin as a control for TT-biotin, and no antigen in the case of RV VLPs. Numbers in each quadrant are the percentage of CD20+, CD27+ or CD27− B cells.

infection and can thus be truly considered mBc (Weller et al., 2005, 2008). Against this possibility is our recent finding that these cells seem to be present in children who do not have RV serum IgA, and thus, by current standards, would be judged to have not been previously infected by RV (Rojas et al., 2007). However, binding of the RV-2/6-VLPs to IgM mBc is specific (inhibited with non labeled antigen (Parez et al., 2004) and MF unpublished data), and antibodies produced by cloned CD27+, IgD+ VLP binding B cells have been shown to be VP6 specific (Tian et al., 2008). The binding of these antibodies to VLPS is probably of low affinity (Kallewaard et al., 2008), but is probably not due to the fact that they are polyreactive, since IgM mBc are not enriched (relative to the IgD− subset) in polyreactive mBc (Tiller et al., 2007). We thus hypothesize that RV VP6, as presented on VLPs, is a member of the group of viral antigens that bind an important part of the repertoire of human Ig, probably as a means to modulate the immune response (Hangartner et al., 2006). The role of such antibodies in the antiviral response of humans has yet to be defined.

Very little is known of the CD27− mBc (Fecteau et al., 2006; Wei et al., 2007; Wirths and Lanzavecchia, 2005). This is the first time that RV-specific CD27− mBc have been studied in detail. We found that the correlation between RV CD27+, IgA mBc and RV plasma IgA levels was lost when the analysis was done on RV CD27+ plus CD27− IgA cells and RV plasma IgA (Fig. 5b). Moreover, the correlation between TT CD27+, IgG mBc and TT plasma IgG (Fig. 5b) was slightly higher than when CD27+ and CD27− subsets were analyzed together. These results suggest that CD27− mBc (specially the RV IgA subset) are not directly related to the levels of plasma Ig. Finally, our results showing little, if any, TT CD27− mBc in our volunteers are in agreement with the fact that TT antibodies are mainly of the IgG1 subclass, which is not highly represented in the CD27− mBc (Fecteau et al., 2006; Wirths and Lanzavecchia, 2005). The relatively high fraction of RV IgG and IgA mBc in the CD27− subpopulation in adult volunteers is in agreement with our previous study of RV mBc in recently infected children (Jaimes et al., 2004). More studies, using recently described markers (Wirths and Lanzavecchia, 2005), are needed to better characterize this population of RV mBc and clarify their function in RV infections.

The levels of total mBc detected by LDA (Fig. 2a) are comparable to those reported by other groups (Amanna and Slifka, 2006; Lanzavecchia, 1983), and show a predominance of IgG, over IgM, over IgA. The levels of total mBc detected by FCA are also comparable to those reported by other groups (Johansson et al., 2005; Tian et al., 2007), where a trend of predominance of IgM, over IgG, over IgA (Fig. 2a) was observed. Moreover, mBc detected by LDA are lower than by FCA, especially for IgM and IgA mBc (Fig. 2). Similar to total mBc, RV IgM mBc were the predominant mBc detected by FCA, but RV IgG mBc predominated by LDA. This is in contrast with the results with TT, for which IgM mBc predominated by both methods. The discrepancies between the two assays have been noted previously (Sasaki et al., 2007) and could be due to at least two non mutually exclusive reasons. First, as noted previously under comparable conditions (He et al., 2004; Lanzavecchia, 1983; Werner-Favre et al., 2001), it is possible that IgM mBc are switching *in vitro* in the LDA to become IgG mBc. Second, it seems possible that not all mBc detected by FCA can be effectively stimulated by CpG/IL-2 to differentiate into ASC (Fig. 2).

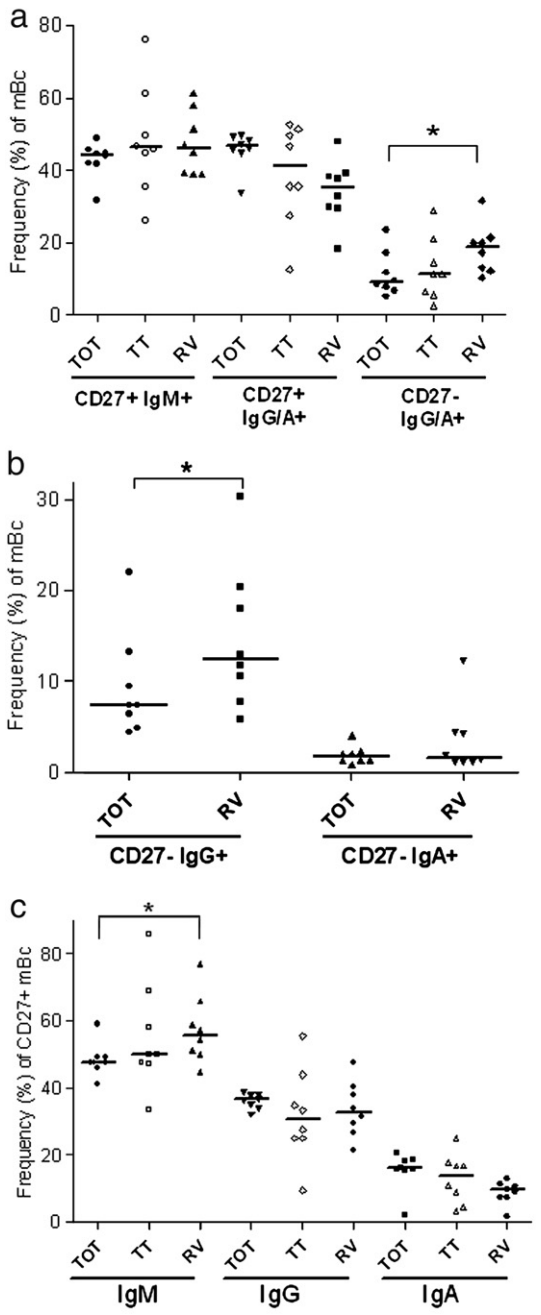

Fig. 4. Frequencies (%) of mBc subsets considering (a) total mBc; (b) total and RV CD27−, IgG+ and IgA+ mBc and (c) CD27+ mBc. *Significant differences (Wilcoxon test, $p<0.05$). Median values are represented by a line.

Perhaps RV IgM mBc (continuously stimulated by RV, a relatively ubiquitous pathogen) represent a population of mBc that are more susceptible to switching *in vitro*. On the other hand, the TT IgM mBc probably represent cells that have not seen their cognate antigen for many years and thus, they may be less prone to switch *in vitro*. In any case, it seems that the functional LDA and non-functional FCA measure overlapping, but partially distinct, subsets of mBc.

To our knowledge, this study is the first to report a relationship between antigen specific circulating IgA mBc and specific IgA levels in plasma. Although RV infection and vaccination stimulate both systemic and intestinal immune responses (Franco et al., 2006), it is probable that the great majority of the RV plasma IgA we measured in healthy adults was produced by non-mucosal IgA secreting B cells, since this IgA does not have a bound secretory component (data not shown) (Brandtzaeg, 2007). Because IgA and IgG ASC secrete similar quantities of Ig (Lanzavecchia, 1983), the relatively higher mean ratio of RV-specific IgA mBc per μg/ml of RV plasma IgA, compared to the TT-specific IgG per mBc/μg/ml TT plasma IgG, could be explained by the shorter half-life of IgA versus IgG (approximately 7 days for IgA *vs.* 20 days for IgG). Thus, circulating RV IgA mBc seem to have the same relationship with plasma IgA as TT IgG mBc have with plasma IgG. Altogether, these results suggest that in the steady state, most circulating RV IgA mBc are systemically derived, and do not come from the intestine. This interpretation is in agreement with the fact that in healthy adults very few circulating IgA mBc express CCR9 (Johansson et al., 2005), a key intestinal homing receptor, and the fact that RV mBc that express CCR9 represent only 1/3 of specific mBc circulating 4 weeks after infection in children (Jaimes et al., 2004). In addition, it has recently been shown that parenteral immunization, but not intranasal immunization, with influenza vaccines induce circulating specific mBc (Sasaki et al., 2007), also supporting the conclusion that mBc in the circulation of healthy adults are predominantly derived from systemic antigen exposure.

It has been proposed that mBc (continuously but non-specifically stimulated to become ASC), recently activated short lived ASC, and long lived bone marrow resident ASC maintain serological memory (levels of specific serum Ig) (Hofer et al., 2006; Lanzavecchia et al., 2006). Of note, we did not find a correlation between the number of circulating total IgM, IgG and IgA mBc and the corresponding levels of plasma Ig (data not shown). We also did not find a correlation between total plasma IgG and IgA and circulating ASC evaluated by ELISPOT in our volunteers ($n=5$) (data not shown). Hence, our results suggest that bone marrow resident ASC maybe an important source of total plasma Ig in the steady state. However, certain subsets of circulating antigen specific mBc (like TT IgG and RV IgA mBc) may also be involved in directly maintaining serological memory, or indirectly linked to this phenomenon, as they correlate with their respective levels of plasma Ig (Fig. 5).

We used TT as a model antigen to compare with RV mBc, and in general our results are in agreement with the literature: the frequency of TT-specific IgG mBc evaluated by LDA (Fig. 2b) is similar to that previously reported using alloreactive T cell clones (Lanzavecchia et al., 1983), and CpG plus IL-2 (Bernasconi et al., 2002) for polyclonal B cell stimulation. The frequency of IgG TT-specific mBc detected by FCA is also comparable to previously reported results using a different FCA (Amanna and Slifka, 2006; Leyendeckers et al., 1999). However, the frequency of LDA TT-specific IgM mBc (Fig. 2b) is about 20 times higher than the frequency previously reported (Lanzavecchia et al., 1983) using alloreactive T cell clones as a stimulus. In addition, previous studies using FCA (Leyendeckers et al., 1999) found that most (68%) TT mBc expressed IgG and not IgM, as opposed to our findings (Fig. 2b). We do not have a clear explanation for these discrepancies. However, it is possible that in the case of the LDA, alloreactive clones are less efficient than CpG/IL-2 at stimulating TT IgM mBc. On the other hand, the mBc detected by the TT Fragment C (an immunodominant recombinant fragment of TT), used previously by other authors (Leyendeckers et al., 1999), could be recognizing a different population of TT mBc than the complete TT antigen used in our study. The correlation between TT IgG mBc measured by LDA and the levels of TT IgG in plasma (Fig. 5) is in agreement with previous findings (Bernasconi et al., 2002). Of note, these results do not necessarily

Table 1
Plasma concentration (μg/ml) of total and antigen specific Ig

	IgM	IgG	IgA
Total	1,367 (649–2,815)	7,390 (6,000–16,000)	1,262 (800–2,825)
RV	ND	0.98 (0.4–3.9)	0.08 (0.013–0.35)
TT	ND	1 (0.48–4.14)	0.02 (0.02–0.04)

ND: not determined. Shown is the median (range) of $n=8$–14 individuals.

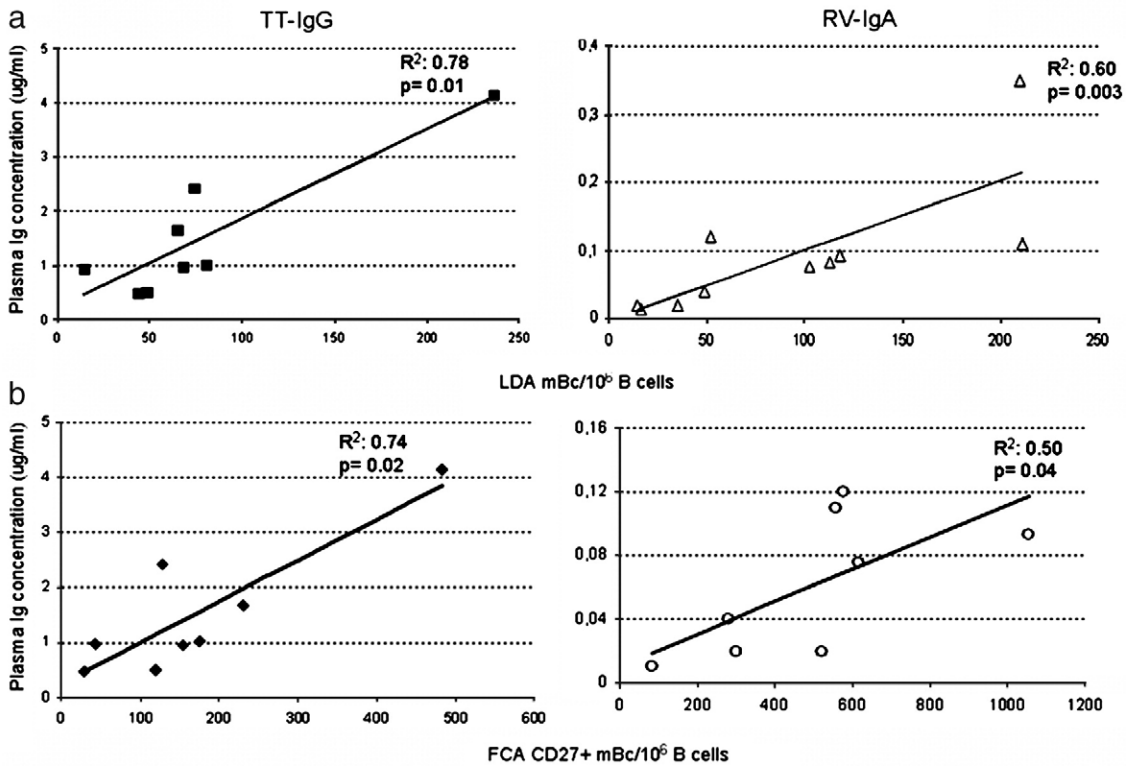

Fig. 5. Correlations between plasma antibody concentrations and frequency of mBc. (a) Significant correlations were found between TT-specific IgG or RV-specific IgA mBc determined by LDA and their corresponding TT-specific IgG, and RV-specific IgA levels in plasma ($n=8$–10, Spearman test, $p<0.01$). (b) Significant correlations were found between plasma antibody levels and the frequency of CD27+ mBc detected by FCA for TT-specific IgG and RV-specific IgA ($n=8$, Spearman test, $p<0.04$). After Bonferroni correction for multiple comparisons the correlations between LDA, but not FCA mBc, and plasma Ig are still significant ($p<0.01$).

contradict other studies (Amanna et al., 2007) in which this correlation was not found, but in which the concentration of plasma TT IgG was measured as a titer and not in μg/ml, as we have done. However, the correlation between TT IgG mBc determined by FCA and TT IgG in plasma (Fig. 5b) is not in agreement with other previous findings (Leyendeckers et al., 1999), probably due to differences in the cell staining method, and as mentioned previously, the fact that the TT antigen used in the previous study was different from the preparation used here. The relatively low levels of TT-specific IgA mBc we found are compatible with the levels of TT IgA ASC induced by TT vaccination (Lue et al., 1994).

In conclusion, we have shown that RV mBc are enriched in CD27+, IgM+ and CD27−, IgG+ mBc (Fig. 4) and that circulating RV-specific IgA mBc (evaluated by both FCA and LDA) correlate with plasma RV IgA concentration. Future studies are necessary to clarify the relationship between the two mBc assays, and to better characterize the CD27+, IgM+ and CD27−, IgG+ RV mBc. Moreover, our studies contribute to understand the relationship between circulating mBc and serological memory, and enhance our capacity to develop better correlates of protection against RV disease. We recently published the results of a clinical trial in which we hypothesized that IgD− RV mBc that express the intestinal homing receptor CCR9 correlate with protection induced by the Rotarix vaccine (Rojas et al., 2007). However, this correlation was poor and the correlations between the frequencies of mBc in the vaccine recipients and their plasma IgA levels were low or non-existent. Possible explanations for these findings are multiple and include the fact that RV mBc are a heterogeneous group of cells and that these numerous groups are related to RV plasma Ig in different ways. The new information provided in the present manuscript and complimentary results (Narváez et al., manuscript in preparation) support this conclusion and should permit us to focus on specific mBc subsets in our future studies with children to identify correlates of protection for RV vaccines.

Materials and methods

Subjects and sample collection processing

Peripheral blood mononuclear cells (PBMC) were isolated from the buffy coats obtained from 19 healthy adult blood donors by density gradient centrifugation using Lymphosep (ICN Biomedical INC, Irvine, CA). Cells were suspended in complete RPMI-1640 supplemented with 10% fetal calf serum, 20 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 0.1 mM of non essential amino acids and 1 mM of sodium pyruvate (all from Gibco-BRL, Gaithersburg, Md) and 5×10^{-5} M of β-mercaptoethanol (Sigma-Aldrich, Saint Louis, Missouri). Cells were washed twice with complete medium and directly tested in the LDA or FCA. From each volunteer, an additional blood sample was collected in heparin-containing tubes and centrifuged at 250 ×g for 5 min and the plasma was collected and stored at −20 °C.

Production of fluorescent virus like particles (VLPs)

Fluorescent RV VLPs were a kind gift of Annie Charpilienne and were produced using baculovirus expression vectors, as previously described (Charpilienne et al., 2001). Briefly, Sf9 cells were co-infected with 2 recombinant baculoviruses at a multiplicity of infection greater than 5 PFU/cell. One baculovirus expressed RF (bovine RV) VP6 and the other a fusion protein consisting of GFP fused to the N terminus of RF VP2 deleted in the first 92 amino acids. Infected cultures were collected 5–7 days post infection and purified by density gradient centrifugation in CsCl. The protein concentration in the purified VLPs was estimated by the method of Bradford. The VP6 protein on the outer shell of the VLPs is recognized by the majority of RV antibodies in infected animals and humans (Youngman et al., 2002).

TT antigen

TT (Staten Serum Institute, Denmark) was resuspended according to the manufacturer's instructions, and biotinylated using the EZ-Link Photoactivatable Biotin kit (Pierce Biotechnology, Rockford, IL) according to manufacturer's protocol. Briefly, 750 μg of TT protein was incubated with biotin at a molar ratio of 20 mol of dye per mole of protein, for 10 min on ice under a UV (365 nm) lamp and then dialyzed against PBS to remove excess biotin. The optimal concentration of the biotinylated TT for labeling of specific mBc was determined using PBMC from healthy and recently TT vaccinated volunteers, and the specificity of binding was evaluated with a competition assay using non biotinylated TT (data not shown).

Flow cytometry assay

Fresh PBMC were washed once with PBS containing 2 mM EDTA (Sigma-Aldrich), 10% fetal calf serum and then incubated with anti CD19 microbeads (Miltenyi Biotec, Auburn, CA). Immunomagnetic positive selection of CD19+ cells was done according to the manufacturer's instructions. The B cells were over 90% pure. The CD19+ B cells were incubated with the GFP labeled VLPs (0.8 μg/test), TT-biotin (5 μg/test) or without any reagent (negative control) for 45 min in the dark, at 4 °C. The cells were then washed with PBS–0.5% bovine serum albumin (Sigma-Aldrich), 0.02% sodium azide (Mallinckrodt Chemicals, Paris, Ky) (staining buffer) and stained with antibodies against CD20 (allophycocyanin labeled), CD27 (phycoerythrin labeled) (BD Pharmingen, La Jolla, CA) and anti-IgG, IgA, IgM (fluorescein isothiocianate labeled) (Caltag Laboratories, Burlingame, CA) or anti IgA, IgM-biotin (Kirkegaard and Perry Labs, Gaithersburg, MD) or IgG-biotin (Jackson Immunoresearch, West Grove, PA). The biotinylated antigen or antibodies were detected using streptavidin-PerCP (BD Pharmingen). After staining, the cells were washed with staining buffer and fixed with 1% paraformaldehyde (Electron Microscopy Sciences, Washington, PA). At least 800,000 purified B cells were acquired on a FACSCalibur (BD Pharmingen) and the data was analyzed with Cellquest Pro software version 2.1. The estimated limit of detection for antigen specific mBc by FCA was ~30 cells/10^6 B cells, values below this number were assigned a value of 15 cells/10^6 B cells for statistical purposes.

Limiting Dilution Assay (LDA)

LDA was performed using PBMC or purified B cells. For most experiments to determine frequencies of RV mBc, and all experiments to evaluate total and TT mBc, PBMC were stimulated using previously described methods (Bernasconi et al., 2002). PMBC were distributed in serial dilutions from 200,000 to 5 cells in a volume of 200 μl per well using 24 replicate cultures per dilution on 96 U wells culture plates (Costar). Cells were stimulated with 2.5 μg/ml of CpG oligodeoxynucleotide 2006 (Microsynth, Switzerland) and 10 ng/ml of human recombinant IL-2 (R&D systems, Minneapolis, MN) for 10 days at 37 °C/5% CO_2. PBMC without stimulation were used as a negative control and analysis of TT mBc was used as a positive control. After 10 days the plates were centrifuged and the supernatants were stored at −20 °C. The supernatants from the 24 replicate cultures were thawed and tested for the presence of total Ig, RV or TT-specific IgG, IgA and IgM by ELISA. The number of PBMC per culture was plotted against the natural logarithm of the fractions of negative cultures for each Ig, as previously described (Lanzavecchia, 1983). Least-squares linear regression was used to fit a line with the different frequencies of negative cultures and the origin. The R^2 (>0.75) and p-value ($p<0.05$) were calculated for each best fitting line. The frequency of mBc producing total or specific antibodies were calculated from the 37% interpolation of the titration curve, that according to the Poisson distribution represents the number of wells containing a single precursor. The frequency of CD20+ cells in fresh PBMC of each volunteer (determined by FCA) was used to express final results in terms of mBc/total B cells. The estimated limit of detection for antigen specific mBc by LDA was ~28 mBc/10^6 B cells and values below this limit were assigned a value of 14.2/10^6 B cells for statistical purposes.

In some LDA experiments PBMC were stimulated, as previously described (Crotty et al., 2004; Sasaki et al., 2007), with CpG (6 μg/ml), 1/10,000 dilution of fixed Staphylococcus aureus Cowan (SAC) (Sigma-Aldrich) and 1/100,000 dilution of pokeweed mitogen extract (SAC and PWM, were kindly provided by Dr. R. Ahmed, Emory University, Atlanta, GA) and cultured as described above.

For LDA with purified B cells, CD19+ cells were positively purified (purity>94%) using microbeads (Miltenyi Biotec). B cells were distributed in serial dilutions from 50,000 to 325 cells in a volume of 200 μl per well, using 24 replicate cultures per dilution in 96 U well culture plates and stimulated with CpG+IL-2 (Bernasconi et al., 2002), CpG+SAC+PWM (Crotty et al., 2004) (as described above for the PBMC), CpG+IL-2 plus purified autologous CD4+ T cells, and CpG+IL-2/IL-6/IL-10 plus NIH/3T3 murine fibroblasts (Amanna and Slifka, 2006). For the last condition 10 ng/ml of hrIL-6 (R&D, Minneapolis, MN) and 15 ng/ml of hrIL-10 (from Pharmingen) were used. The feeder cells for the last two conditions (microbead (Miltenyi Biotec) positively selected autologous CD4+ T cells (purity>98%) and NIH/3T3 murine fibroblasts (a gift from E. Butcher, Stanford University CA), respectively) were treated with 50 μg/ml of mitomycin C (Sigma-Aldrich) for 30 min, washed exhaustively and then used at 5000 cells/well. After 10 days of culture, the supernatants were collected to evaluate RV IgM/IgG/IgA by ELISA, as described below.

ELISA for measuring RV and TT-specific antibodies and total Ig in plasma and LDA culture supernatants

Antigen specific IgA, IgG and IgM were determined in plasma and culture supernatants by ELISA, as previously described (Gonzalez et al., 2003; Lanzavecchia et al., 1983), with some modifications. For total IgG, IgA, IgM, TT-specific IgG, IgA, IgM and RV-specific IgG and IgA, Immulon 2 ELISA plates (DYNEX Technologies, Chantilly, VA) were coated with appropriate concentrations of anti whole Ig (Sigma-Aldrich), TT, VLPs, or PBS (negative control) and incubated overnight at 4 °C. After discarding these solutions, 150 μl 5% blotto was added to the plates and incubated at 37 °C for 1 h. Then, the blotto was discarded and 70 μl of the dilutions of plasma in 2.5% blotto or the culture supernatants were deposited in each well. After 2 h incubation at 37 °C, the plates were washed three times with PBS-Tween 20 and 70 μl of biotin-labeled goat anti-human IgA, IgG or IgM (Kirkegaard and Perry Labs) diluted in 2.5% blotto was added and incubated for 1 h at 37 °C. After three washes with PBS-Tween, 70 μl streptavidin-peroxidase (Kirkegaard and Perry Labs) 1/1000 dilution in 2.5% blotto was added and the plates were incubated for 1 h at 37 °C. After three washes with PBS-Tween 20, plates were developed using 70 μl tetramethyl benzidine substrate (TMB, Kirkegaard and Perry Labs). The reaction was stopped by the addition of 17.5 μl of 2 M sulfuric acid. Absorbance was read at a wave length of 450 nm on an ELISA plate reader.

For detection of RV-specific IgM, 96 well plates were coated overnight with 70 μl of Goat $F(ab')_2$ (0.7 μg/well) anti-human IgM (Biosource International, Camarillo, CA). The plates were then blocked and incubated with plasma or supernatant culture samples, as described above. After three washes with PBS-Tween 20, 70 μl of VLPs (16 ng/well) were added to individual wells and incubated for 1 h at 37 °C. Plates were then washed and guinea pig anti-rhesus RV hyperimmune serum was added and incubated for 1 h at 37 °C. After three washes, 70 μl of biotin-labeled goat anti-guinea pig (Vector Laboratories, Burlingame, CA) in 2.5% blotto-1.5% normal goat serum was added and incubated for 1 h at 37 °C. After three washes with

PBS-Tween, 70 µl streptavidin-peroxidase was added, and finally, the assay was developed as described above.

A standard curve was constructed using known amounts of purified total IgG, IgA (Sigma-Aldrich) and purified RV-specific IgG and IgA or TT-specific IgG and IgA. The antigen specific Igs were purified using a CarboxyLink kit (Pierce Biotechnology). Briefly, RV-DLPs or TT antigen, were fixed to the column and the purified IgG or IgA (Sigma-Aldrich) bound to the antigens and latter eluted from the columns according to manufacturer's instructions. Ig concentrations in plasma were calculated using the program ELISA for Windows, Version 2.00 (http://www.cdc.gov/ncidod/dbmd/bimb/elisa.htm). The limits of detection for each plasma ELISA were as follows: 3 ng/ml for total IgA, 4 ng/ml for total IgG, 12.5 ng/ml for RV IgA, 46 ng/ml for RV IgG, 20 ng/ml for TT IgA and 52 ng/ml for TT IgG. The purified RV IgG and IgA did not recognize TT and the purified TT IgG and IgA did not recognize RV (data not shown).

The culture supernatants assayed by ELISA were scored as positive if they had an optical density exceeding 0.1 and the optical density was twice the optical density of wells treated with PBS. We used standard serial dilutions of known plasma as a positive control in each plate. To be accepted for analysis, the titer of the positive control plasma in a plate could not differ by more than one dilution from plate to plate.

Statistical analysis

Analysis was performed with SPSS software version 10.0 (SPSS Inc., Chicago, IL). Differences between groups were evaluated with a Wilcoxon test, and correlations between the different variables were evaluated with two tailed Spearman's tests. Significance was established if $p \leq 0.05$. Data are shown as medians and ranges unless otherwise noted.

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